

## STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHEDS): A NEW HORIZON

JAIGANESH. I<sup>1</sup>, REVATHY. S<sup>2</sup>, DHARMA. R. M<sup>3</sup>, NAGARATHNA. C<sup>4</sup> & SHAKUNTALA. B. S<sup>5</sup>

<sup>1</sup> Senior Lecturer, Pedodontics and Preventive Dentistry, Thai Moogambigai Dental College and Hospital,  
Dr.MGR University and Research Center, Chennai, Tamil Nadu, India

<sup>2</sup> Senior Lecturer, Pedodontics and Preventive Dentistry, The Oxford Dental College and Hospital,  
Bangalore, Karnataka, India

<sup>3</sup> Professor, Department of orthodontics, DAPMRV Dental College and Hospital, Bangalore, Karnataka, India

<sup>4,5</sup> Professor and Head, Department of Pedodontics and Preventive Dentistry, Rajarajeswari Dental College  
and Hospital, Bangalore, Karnataka, India

### ABSTRACT:

**Background and Objectives:** Stem cells from human exfoliated deciduous teeth (SHED) have been demonstrated as a novel population of adult stem cells capable of multi-differentiation potential. Hence, isolating and evaluating the pluripotency of SHEDs opens a new era in the field of dentistry for the treatment of damaged teeth.

The present study was conducted to isolate and differentiate Stem cells from Human Exfoliated Deciduous teeth.

**Method:** Study samples comprise of 30 extracted exfoliated primary teeth collected from children aged 6 to 14 years. Isolation of SHEDs was done followed by which flowcytometric analysis and tri-lineage differentiation was performed. Growth kinetics of SHEDs were assessed.

**Results:** SHEDs were successfully isolated. Flowcytometric analysis of SHEDs showed expression of positive markers CD73, CD90, CD105 while no expression of negative markers CD34, CD45 and HLA-DR. SHEDs differentiated markedly into osteocytes, chondrocytes while less significant differentiation of adipocytes were observed. The Population Doubling was found to decrease with increase in passage number while Cumulative Population Doubling was found to increase with increase in passage number. Time Doubling was found to be 14 hours which means that SHEDs have high proliferation potential.

**Conclusion:** Stem cells from Human Exfoliated Deciduous teeth are mesenchymal stem cells which are multipotent and can serve as a promising incentive for therapeutic and future research purposes.

**KEYWORDS:** Mesenchymal Stem Cells, Deciduous Teeth, Tissue Engineerin, Dental Pulp

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### INTRODUCTION

Human cells and tissues have a capability for regeneration. Cells like erythrocytes and keratinocytes are regenerated very quickly, whereas some cells like visceral epithelial cells proliferate slowly. However, most of the cells in animal tissues regenerate or proliferate continuously although there are some disparities in their proliferative capacity. Certain types of undifferentiated cells that proliferate and redevelop constantly throughout

life are responsible for the regenerative capacity of the tissues. Some cells have the ability to differentiate not only into cells of their original tissues but also into other cell lineages.<sup>1</sup> Such type of clonogenic cells capable of both self-renewal and multilineage differentiation with varying degrees of potency and plasticity are known as stem cells.<sup>2</sup>

Stem cell is a broad term used to describe a wide variety of cells from varying sources. Stem cells can be divided into two categories – embryonic and adult or postnatal. Embryonic stem cells are totipotent cells capable of differentiation into virtually any cell type, as well as being propagated indefinitely in an undifferentiated state. However, embryonic stem cells have both moral and ethical problems - as these cells will later develop into a human being, taking these cells will require the destruction of an embryo. Technically these cells are difficult to control and might grow as well to form tumors after their injection.<sup>3</sup> Adult stem cells are not totipotent, and they can be further classified depending on their origin and differentiation potential.

Adult stem cells have been isolated from a variety of sources which includes bone marrow, brain, skin, hair follicles, skeletal muscle and especially dental tissues.<sup>4</sup> Dental stem cells are considered to be an appealing source for mesenchymal stem cells, since they are non-controversial, readily accessible, have a large donor pool, and pose no risk of discomfort for the donor.<sup>5</sup>

Stem cells from human exfoliated deciduous teeth (SHEDs) represent a population of postnatal stem cells capable of extensive proliferation and multipotential differentiation. The transition from deciduous to permanent teeth is a very unique and dynamic process in which the development and eruption of permanent teeth synchronize with the resorption of the roots of deciduous teeth. An exfoliated deciduous tooth is similar in some ways to an umbilical cord, containing stem cells that may offer a unique postnatal stem cell source for potential stem cell based clinical applications.<sup>6,7</sup>

The complex structural composition of teeth provides hardness and durability, these rigid structures are vulnerable to damage caused by mechanical trauma, chemicals, congenital defects, cancer and bacterial infections. Unlike other tissues such as bone, which have the capacity to repair and remodel throughout post-natal period, the relative static components of teeth do not readily undergo complete regeneration following insult. However adult teeth do demonstrate some limited reparative process such as the formation of tertiary or reparative dentin which has more poorly organized mineralized matrix compared to primary and secondary dentin, that serves as a protective barrier to the dental pulp.<sup>8</sup> Similarly, the periodontium has limited capacity for regeneration. Despite our extensive knowledge concerning the pathology of diseases of teeth, restorations of damaged or diseased dental tissues, to date, has relied primarily on the use of synthetic implants and structural substitutions comprised of inert compounds.<sup>9</sup> However, it was identified that putative dental stem cell populations capable of regenerating organized tooth structures has stimulated interest into the potential use of post-natal stem cell based therapies to treat the damage caused by trauma, cancer, caries and periodontal diseases.<sup>10,11</sup>

Hence, isolating and evaluating the pluripotency of SHED opens a new era in the field of dentistry for the treatment of damaged teeth. Further, there is lack of substantiating studies on stem cells from naturally exfoliating primary teeth which overcome the disadvantages of other methods of obtaining stem cells and ethical issues.

## **MATERIALS AND METHODS**

The study was conducted in the Department of Pedodontics and Preventive Dentistry, Rajarajeswari Dental College and Hospital in collaboration with Manipal Institute of Regenerative Medicine, (Stempeutics) Bangalore.

Institutional ethical committee approval was obtained from the Ethical Committee, Rajarajeswari Dental College

and Hospital. A written informed consent was obtained from the parents/ guardian of the children included in the study.

Thirty exfoliating primary teeth from normal healthy children aged between 6 to 14 years and Primary teeth extracted for orthodontic correction were included in the study. Children with special health care needs, teeth with developmental anomalies, traumatized primary teeth and teeth with caries or any associated pathology were excluded from the study.

### **Sample Collection**

Primary teeth were then extracted and rinsed with normal saline gently to remove blood and other debris. The samples collected from each individual were then separately transferred to 15ml conical base centrifuge tubes (BD Falcon) containing Dulbecco's Phosphate Buffer Saline (DPBS) solution (Invitrogen). The specimen is transferred to Manipal research center within 2 hours.

### **Isolation of SHEDs**

In the laboratory, the samples were processed inside the Bio-laminar flow chamber. First, the samples were washed with PBS + 1% antimycotic twice. Then teeth were placed inside a sterile surgical glove and broken into pieces with an osteotome wrapped with aluminium folds, so that the pulpal tissues can be easily removed. The pulpal tissues were then removed with broaches or tweezers and placed in 50mm x 12mm petri dishes. Followed by which, mincing was done using 2mg/ml collagenase blend (Sigma) and the tissues were cut in to smaller pieces using Surgical Scalpel Blade no. 21 to increase the surface area of action to the enzyme. The tissue was incubated in the incubator (Heracel Thermo) at 37°C for 60 minutes. After incubation, the culture medium, Dulbecco's Modified Eagles Medium- Knock out (DMEM-KO) with 10% Fetal Bovine Serum (Hyclone), 100µM ascorbic acid and 2mM L-Glutamax supplemented with 100U/ml penicillin and 100U/ml streptomycin, was added. The samples were transferred to BD falcon tubes and then centrifuged (Eppendorf Centrifuge Machine 5415R, Germany) at 1800 rpm for 5 minutes. The supernatants were discarded and the tissue pellets were collected in 6 well culture plate which appropriately containing 1ml of culture medium, DMEM-KO. The cells were finally incubated in the incubator (Heracel Thermo) in a humidified atmosphere at 95% air and 5% CO<sub>2</sub> at 37°C. The plates were then reviewed after 48 to 72 hours to check for growth and attachment of cells.

### **Cell Passaging**

Media was changed every 48 hours. Several representative microphotographs were taken each time during observation with inverted microscope at 4x and 10x magnification. Once the cells become 80% confluent, the spent media was removed and cells were washed by DPBS and detached by adding 0.25% trypsin. After neutralization, the cells were transferred into a centrifuge tube and centrifuged at 1800 rpm for 5 minutes. Then cells were reseeded in larger containers passage to passage progressively using petri dish and 35mm<sup>2</sup> flask.

### **Characterization of SHEDs**

#### **Flowcytometry**

Flowcytometric analysis was used to determine the surface phenotypic profile of the isolated cells. Ten microliter of tagged primary antibody were added on appropriate number of cells. Isotopes of Immunoglobulin G2 (IgG2) and Immunoglobulin G1 (IgG1) were used as control groups. Cells were stained for 1hour on ice. Then 500µl of FACS buffer were mixed thoroughly by pipetting and then transferred to flowcytometry tubes. The samples were runned in the flowcytometry machine. The cells were stained using fluoresceinisothiocyanate (FITC) –conjugated CD34, CD105 and PE

(R- phycoerythrin)-conjugated CD45, CD90, CD73 and HLA-DR antibodies. The process was repeated in 10 samples for each marker. BD CellQuest™ Pro Version 5.2.1 software (BD Bioscience, USA) was used to analyze the flow cytometric results.

**Table 1: Types of Markers Studied to Characterize SHEDs**

Positive markers	CD 73, CD 90, CD 105
Negative markers	CD 34, CD 45, HLA-DR

**Table 2: Isotypes, Antibodies and Stains Standardized for Flowcytometry Experiment**

Antibody	Stain	Isotype tagged
CD 34	FITC	IgG1
CD 45	PE	IgG1
CD 73	PE	IgG1
CD 90	PE	IgG1
CD 105	FITC	IgG1
HLA DR	PE	IgG2A

### Tri-Lineage Differentiation

Cell lineage induction was performed when cultures had reached 90% confluency. SHEDs were cultured in complete media supplemented with the respective induction media in 35 mm<sup>2</sup> dish (Table – 4, 5). Media was replenished every 3 days. After 7 to 21 days, cells were fixed to do Von Kossa staining for osteocytes, Oil red O for adipocytes and Alcian Blue Stain Analysis for chondrocytes. The differentiation induction was repeated in 3 samples for each lineage.

**Table 3: Media Composition for Osteocytes Differentiation**

Reagents	Concentration
Basal media (DMEM-KO)	
FBS	10%
Pen – strep	200 mM
Dexamethasone	0.5%
Ascorbic acid	50 µgm/ml
B-glycerophosphate	10 Mm

**Table 4: Media Composition for Adipocytes Differentiation**

Reagents	Concentration
Basal media (DMEM-KO)	
FBS	10%
Pen – strep	0.5%
Glutamin	200 Mm
Dexamethasone	1 µM
Insulin	1 µgm/ml
Indomethacin	100 µM
IBMX	0.5 mM

STEMPRO Chondrogenesis Differentiation Kit (Invitrogen) was used for chondrocytes differentiation.

### Growth Characteristics of the Cells

**Population doubling (PD):** By expansion of SHEDs, cells were counted at the end of each passage once they were 85% to 90% confluent. Population doubling (PD) was determined by using the following formula:

$$PD = [\text{Log}N_t - \text{Log}N_0] / \text{Log}2$$

where  $N_0$  is the initial number of cells and  $N_t$  is the final number of cells at each passage.

**Cumulative Population Doubling (CPD):** CPD was calculated by addition of the population doubling for each passage to the population-doubling level of the previous passage.

$$CPD = [\text{Log}_{10} (N_t/N_0)] * 3.33$$

$N_0$  is the initial number of cells and  $N_t$  is the final number of cells at each passage.

**Population Doubling Time (PDT):** It is defined as the time by which a given cell population doubles in number. It is indeed an indication of the rate of proliferation of the given cell population in culture. Time doubling (TD) has been calculated by 24 well plate experiments.

Cells were plated in each well of the 24-well plate (10000 cells/cm<sup>2</sup>). Cells in 3 wells were counted at a time after 24, 48, 72 hours and so on till the 192<sup>nd</sup> hour (8<sup>th</sup> day). Values were plotted on a graph, as cell number (cells/ml) against time (days).

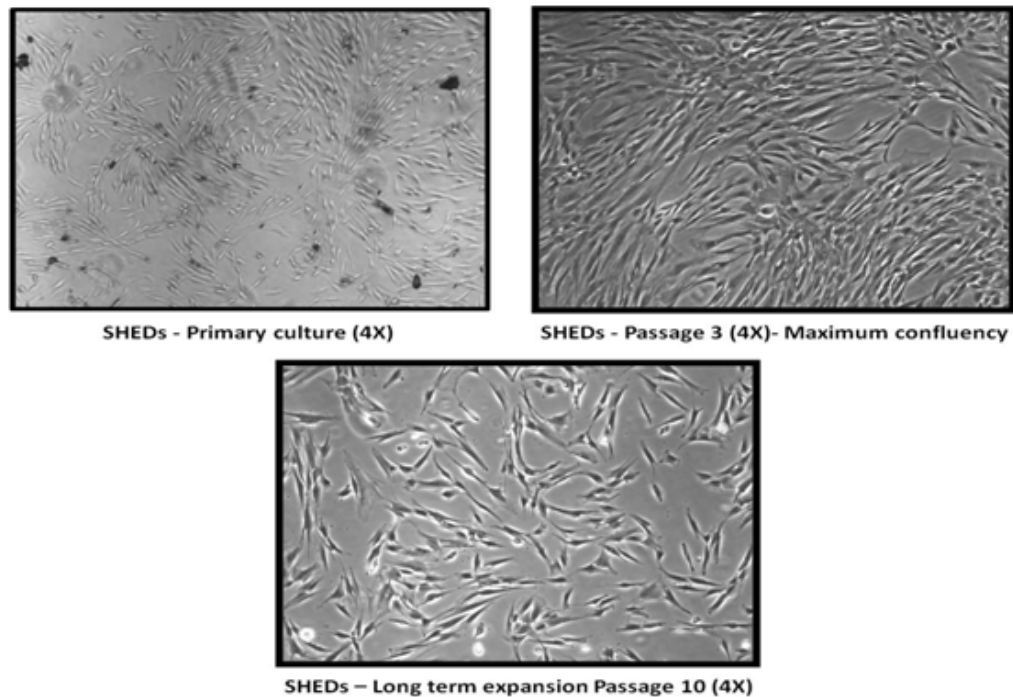
### Statistical Analysis

The data was analysed by calculating z-test for proportions regarding sample distribution. Flowcytometric analysis of cell surface marker expression was analysed by BD Cell Quest<sup>TM</sup> Pro Version 5.2.1 software and expressed as arithmetic mean  $\pm$  Standard Deviation (SD).

## RESULTS

### Cell Culture

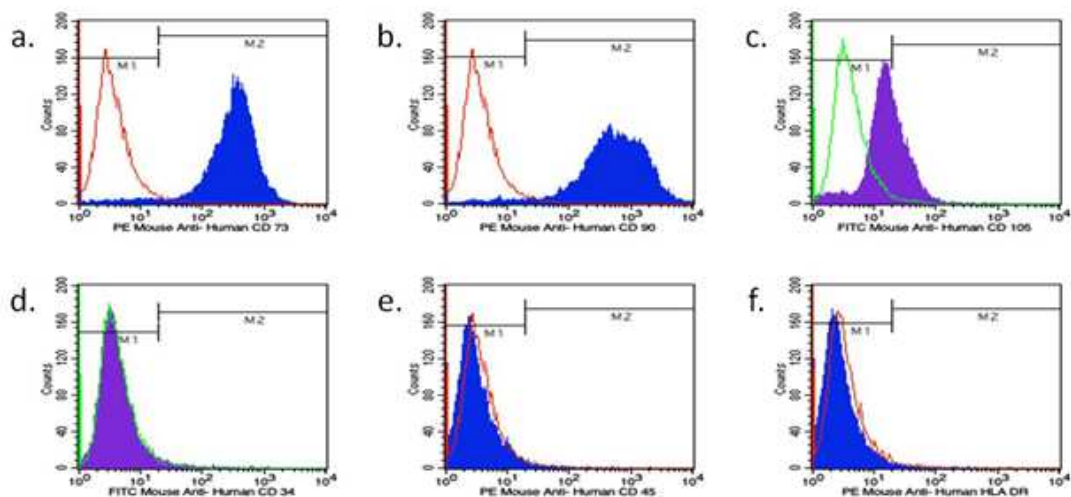
After 24 to 48 hours of cultivation, SHEDs were observed as single cell or as small colonies. Dental pulp cells were observed to grow with colony formation at primary culture. The colonies were mainly composed of fibroblastic cells. A number of small clear cells were also observed on the fibroblastic cells. The cultures tended to achieve confluence in 10 days. Confluent cultures were composed of multiple bundles of fibroblastic cells each running in a particular direction, a feature typical of human marrow-derived Mesenchymal Stem Cell culture. (Figure – 1)



**Figure 1: Inverted Microscopic Images at Different Passages**

### Flowcytometric Analysis

Flowcytometric analysis of SHEDs showed high expression of positive marker CD73 (96.69 %), CD90 (97.70 %) and moderate expression was observed for CD105 (34.33 %). SHEDs progeny did not express negative markers CD34 (1.76%), CD45 (0.88%) and HLA-DR (0.58%). These values were processed by BD Cell Quest™ Pro Version 5.2.1 software and graphs were generated.



**Figure 2: Flowcytometric result showing positive expression for CD 73, 90 (a,b), moderate expression for CD105 (c) and negative expression for CD 34,45 & HLA-DR.(d, e, f)**

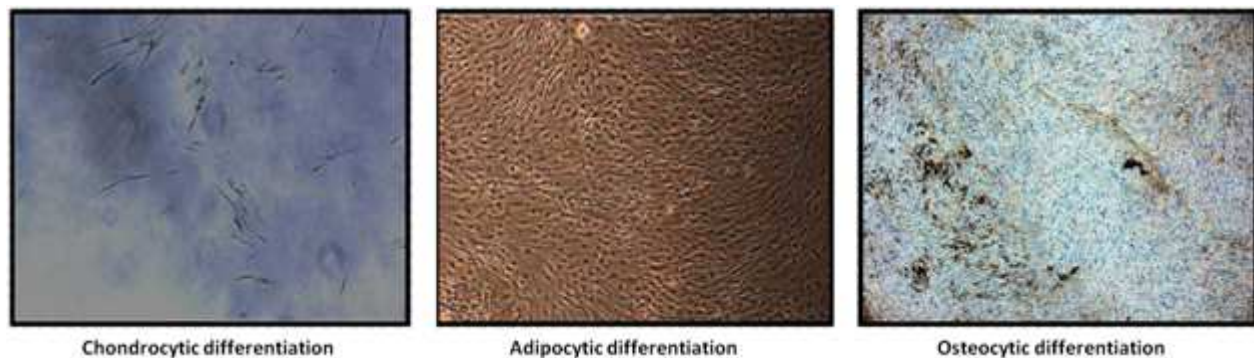
### Tri-Lineages Differentiation

**Chondrocytes:** Chondrogenesis was detected by the presence of proteoglycans stained with Alcian Blue staining

at day 21. SHEDs were shown to have relatively good chondrocytic differentiation potential.

**Adipocytes:** Adipogenesis was detected by neutral oil droplet formation stained with Red O at day 21. The potential of adipogenic differentiation in SHEDs appeared to be weak, since a few cells per microscopic field were observed to be differentiated into adipocyte in differentiation culture.

**Osteocytes:** Osteogenesis was confirmed by mineralized matrix deposition stained with Alizarin Red at day 21. SHEDs differentiated into osteocytes remarkably.

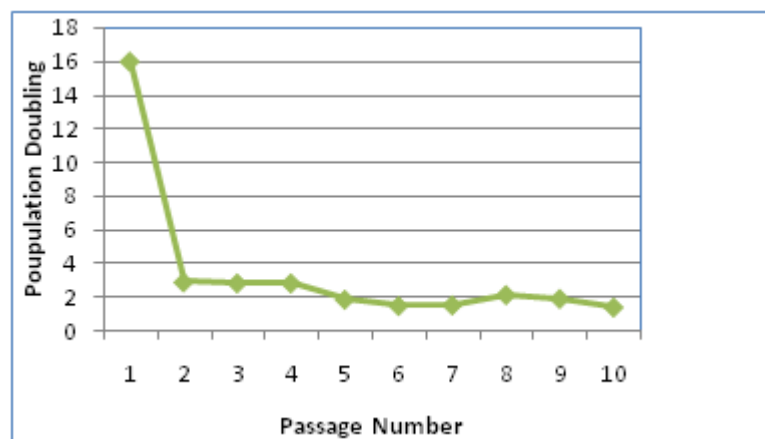


**Figure 3: Trilineage Differentiation**

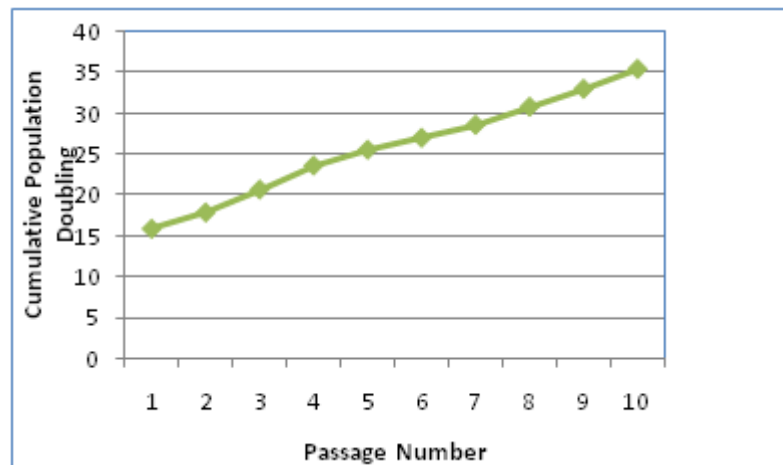
### Growth Kinetics

#### Population Doubling and Cumulative Population Doubling

The Population Doubling was found to decrease with increase in passage number while Cumulative Population Doubling was found to increase with increase in passage number.



**Graph 1: Plot of Average Population Doubling against each Passage Number. Population Doubling was Found to Decrease with Increase in Passage Number**



**Graph 2: Plot of Average Cumulative Population Doubling Against each Passage Number. Cumulative Population Doubling Increases with Increase in Passage Number**

## DISCUSSIONS

Mesenchymal stem cells from human exfoliated deciduous teeth have become an attractive alternative in the field of tissue engineering. The use of Stem cells from Human Exfoliated Deciduous teeth (SHEDs) might bring advantages for tissue engineering over the use of stem cells from adult human teeth, as SHEDs were reported to have higher proliferation rate and increase cell population doublings as compared with stem cells from permanent teeth.<sup>6</sup>

Recent findings demonstrated that the mesenchymal progenitors isolated from the pulp of human deciduous incisors, SHEDs (Miura *et al.*, 2003) exhibited high multipotency since they could differentiate into adipocytes, osteoblasts and chondroblasts. Moreover, the non immunogenic characteristic of dental stem cells has paved the way for efforts to store the exfoliated deciduous teeth which is usually discarded, for use in the future.

In this study, SHEDs were successfully isolated and identified. We were able to isolate SHEDs mostly from primary anterior teeth due to the more feasibility of using barbed broach in single rooted teeth. Another probable reason is the broader root base of primary molars and therefore, are retained in the mouth for a longer period of time, a longer amount of time to resorb, which may result in an obliterated pulp chamber that contains less pulp, and thus, no stem cells.<sup>10</sup>

Phenotypical analysis of SHED in this study showed high positivity for positive markers CD73 (96.69 %), CD90 (97.70 %) and low positivity was observed for CD105 (34.33 %) which is commonly expressed by endothelial progenitors. This is in consistent with other studies.<sup>12,13</sup> The cultivated SHED cells are not hematopoietic, because they did not express negative markers CD34 (1.76%), CD45 (0.88%) and HLA-DR (0.58%). In one of the studies carried out on dental pulp stem cells, the phenotypic expression of CD105 was found to be increased with increase in passage number.<sup>13</sup> The expression was found to be below 40% when the flowcytometry was done using passage, p5 to p6 while the expression increased to more than 55% when p8 to p10 was used.<sup>13</sup> This may be the probable explanation as to why there was moderate phenotypic expression of CD105 in the present study as compared to other positive markers as the flowcytometry was conducted in early passage. In another study, contrary to the present study, SHEDs were found to express CD105 well but an extra incubation period with a secondary mAb (goat FITC-labeled against mouse Abs, Serotec) was carried out in that case.<sup>14</sup> However, in the present study, no such extra incubation was carried out. This differences in the process of flowcytometry might account for the difference in expression of CD105.

Our data demonstrated a clear osteogenic and chondrogenic potential of SHEDs. On the other hand, we were able to trigger weak adipogenesis from dental pulp stem cells. Nevertheless these findings are in agreement with observation of Gronthos et al. and others who expanded dental pulp stem cells from single cell clones and demonstrated their osteogenic properties and no formation of lipid laden adipocytes.<sup>15, 16</sup> However, later study reported that dental pulp stem cells were able to generate adipocytes unwillingly.<sup>17</sup>

When monitoring parameters related to cell kinetics such as Population Doubling, Cumulative Population Doubling and Time Doubling over time, we noticed some characteristic changes in human SHEDs that could be summarized as a decrease of their proliferation rate during long-term cell cultivation and increase of their doubling time. Similar findings were also published by Huang et al. who reported rhesus DPSCs had a high proliferation rate during early passages from establishment of the cell lines which decreased gradually during cultivation.<sup>18,19</sup> Nevertheless, the overall increase in doubling time indicates stem cell ageing that is linked with a decrease of their regenerative capacity and with ageing of tissues.

## CONCLUSIONS

Dental pulp tissue from exfoliated primary teeth represents an easily accessible source of tissue, one which is often discarded, but which instead may be useful as source of stem cells for research and clinical applications. This would open a new perspectives for the treatment of stem cell based therapy of various Dental and Medical diseases

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